HYALURONIC ACID BUTYRIC ESTERS WITH A LOW DEGREE OF SUBSTITUTION, PROCEDURE FOR THEIR PREPARATION AND USE

FIELD OF THE INVENTION

The invention relates to substituted polysaccharide compounds possessing antiproliferative activity.

PRIOR ART

Butyric acid exhibits considerable antiproliferative activity, inhibiting cell growth and inducing differentiation in a wide variety of cells with altered phenotype as demonstrated in experiments on various cell lines of neoplastic origin.

The molecular mechanisms at the basis of butyric acid biological activity are principally linked to the inhibitory action exerted by the compound on the activity of members of the histone-deacetylase family, they being enzymes which, together with histone-acetyl-transferase, regulate gene transcription by modulating the degree of acetylation of histones, proteins closely associated with DNA.

While possessing good clinical application potential, the sodium salt of butyric acid has the disadvantage of a very short half-life which strongly limits its clinical use due to the difficulties in achieving plasma concentrations which are sufficient to provide a therapeutic effect. In this respect, when administered intravenously, sodium butyrate has a half-life of only 5 -6 minutes before being metabolised.

Of the approaches that have been explored for overcoming this problem, the first one to mention is the chemical approach, aimed at stabilizing the molecule by esterification in order to slow down its degradation and prolong its biological activity. Indeed, certain simple esters of butyric acid such as phenyl butyrate are known to exhibit antineoplastic activity in various forms of tumour such as prostate cancer (Proceedings of the American Association for Cancer Research, vol. 37, March 1996, 498). In this case, esterification simply has the purpose of increasing plasma half-life of the active principle, transforming the compound into a prodrug which on hydrolysis induces the slow release of butyric acid into the target organ. The activity of these esters, however, is always lower than that of the free acid due to the reduced bioavailability of the active principle.

An alternative approach has been to encapsulate the molecule within liposome structures capable of providing it with greater protection from degradation and

enabling it to be delivered at higher concentrations (G. Storm et al., Biotherapy, 3:25, 1991); again in this case the results obtained are not satisfactory due to the poor efficiency of entrapment of the liposome vesicles.

EP 0356275 describes glycosaminoglycans in which the hydroxyl and amine groups are substituted by acyl or sulfate functional groups. US 5,185,436 describes instead the preparation of butyric acid derivatives in which the active molecule is covalently bound (ester bond) to a non-toxic carrier. Finally, US 6,140,313 describes esters of hyaluronic acid (HA) with butyric acid obtained by a procedure in which hyaluronic acid is dissolved in H_2O, acidified and precipitated by adding collidine salts. Even though the HA is dissolved in dimethylformamide (DMF), the procedure is not completely anhydrous and the esters obtained have a degree of substitution (ratio of average mole value of butyric acid residues to number of moles of disaccharide units GlcNAc-GlcUA) never lower than 0.1.

SUMMARY

The invention relates to hyaluronic acid esters in which hydroxyl groups of hyaluronic acid are partially esterified with butyric residues and which are characterised by a degree of substitution with butyric residues (ratio of number of butyric acid residues to disaccharide units GlcNAc-GlcUA of hyaluronic acid) being less than or equal to 0.1 These esters with low degree of substitution are obtained by means of a process undertaken in the homogeneous phase under anhydrous conditions, the hyaluronic acid being used in the form of a quaternary nitrogen salt. In one of its preferred embodiments the process comprises the following passages:

a) dissolving a quaternary nitrogen salt of hyaluronic acid at a concentration between 1-100 g/litre in a polar aprotic solvent optionally heated to temperatures above 50°C,

b) preparing the acylating reagent by mixing butyric anhydride and a 4-dialkylaminopyridine in a polar aprotic solvent,

c) adding the acylating reagent to the hyaluronic salt solution under anhydrous conditions,

d) purifying the reaction product or alternatively converting the ester obtained into the corresponding sodium salt by means of acidification.

The esters of the invention have a greater antiproliferative activity than the corresponding esters with a higher degree of substitution, in particular against primary and metastatic tumours, where said tumours are primary of hepatic origin, or are hepatic metastases.

A further aspect of the invention is represented by pharmaceutical compositions, containing as active principle at least one of the esters described.

DESCRIPTION OF THE FIGURES

Figure 1. ^{1}H-NMR spectrum of the hyaluronic acid butyric ester HE2. The DS of HE2 (0.2) was calculated by integration and normalisation of the methyl group signal (δ 0.83 ppm, triplet) of the butyrate residue with respect to the methyl group signal (δ 1.92 ppm, singlet) of the N-acetyl group of the glucosamine residue in the polysaccharide structure.

Figure 2. CE-UV analysis of the hydrolysis products of the hyaluronic acid butyric ester HB4 prepared as in example 4. Buffer used: 50 mM Borax. Attribution of peaks: [1] HA; [2] butyric acid.

Figure 3. Comparison of the antiproliferative effect of graduated doses of hyaluronic acid butyric esters at various degrees of substitution in colon carcinoma (HT29) and hepatocarcinoma (HepB3) cells.

The effect of the esters of the invention of cell proliferation was evaluated by MTT colorimetry and expressed as percentage inhibition compared to the control consisting of cells maintained in culture medium alone.

Panel A: Shows the growth inhibition effect induced by four butyric esters (HE3/T, HB4, HB10 e HB11) compared with sodium butyrate in the HT29 colon carcinoma cell line.

Sodium butyrate (NaB): -■- ; butyric ester with 0.1 d.s. (HE3/T): - ● - ; butyric ester with 0.062 d.s. (HB4): -▲- ; butyric ester with 0.025 d.s. (HB11): -◆- ; butyric ester with 0.004 d.s. (HB10): -▼- .

Panel B: Shows the growth inhibition effect induced by three butyric esters (HB4, HB10 and HB 11) compared with sodium butyrate in cell line HepB3.

Sodium butyrate (NaB): -■- ; butyric ester with d.s. 0.062 (HB4): -▲- ; butyric ester with d.s. 0.025 (HB11): -◆- ; butyric ester with d.s. 0.004 (HB10): -▼- .

Figure 4. Dose-dependent antiproliferative effect of treatment with the esters of

the invention. The ester HB10 (Ha-But) prepared in example 2 compared with sodium butyrate (NaB) (upper panels, as a function of the concentration in mM of butyric residues) or hyaluronic acid (HA) (lower panels, as a function of concentration in mg/ml) was used for treating the HepG2 and HepB3 cell lines for 6 days in an antiproliferative activity assay. In both cell lines HB10 was found to be 10 times more effective at inhibiting cell growth that the active principle, as highlighted by the comparison with IC_{50} values (concentration able to inhibit 50% of cell growth):2.1 vs 0.12 mM for HepG2 and 3 vs 0.31 mM for HepB3.

Figure 5. Evaluation of CD44 membrane antigen expression in hepatocarcinoma HepG2 and HepB3 cell lines (*Panel A*). The analysis was conducted by flow cytometry with a mouse primary antibody directed against human CD44 and with an anti-mouse secondary antibody labelled with FITC. In the figure, the negative control is shown in black (consisting of the sample without primary antibody) and specimen under examination is shown in white.

Panel B: Evaluation of the binding kinetics of ^{99m}Tc-HA-But in HepG2 and HepB3 cell lines. The labelled compound, purified ^{99m}Tc-HA-But diluted with cold polymer until a concentration of 5 mg/ml was obtained, was added to the cells, cultivated in RPMI 1640 with 10% v/v fetal bovine serum, in a chamber-slide and left to react for 6 hours at 37°C. The photograph was taken after 6 hours' treatment.

Panel C: Evaluation of cell uptake with time. The HepB3 cell line, with high expression of CD44, showed greater binding activity than the HepG2 line which barely expressed CD44 and attained saturation after about 6 hours from the start of treatment.

Figure 6. Histological examination of liver sections of animals injected intrasplenically with tumour cells and treated with the esters of the invention. Microscopic images depicting liver histology of animals injected intrasplenically with Lewis carcinoma LL3 cells and either treated or not treated with HA-But. The 4µm thick histological sections were taken from liver samples of treated or control animals, fixed in 10% formalin and enclosed in paraffin. After staining with Cajal-Gallego the sections were examined by optical microscope. In the liver samples derived from animals treated with Ha-But, no sign of any damage to the morphology of the organ, possibly induced by the compound, was noted.

Figure 7. Diagram of Kaplan-Meier survival. The survival analysis of mice intrasplenically inoculated with B16/F10 melanoma cells and treated daily s.c. with HB10 at a dose of 4.8 µmoles/mouse/day with weekly i.p boosters of 12 µmoles/mouse on days 4, 11, 18, 25, 31 was evaluated using the Kaplan-Meier method. After 90 days' observation, 8 out of 10 of the treated animals were still alive, as opposed to only 3 out of 11 of the control animals.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to hyaluronic acid butyric esters with a degree of substitution (ratio of average mole value of butyric residues to number of moles of disaccharide units GlcNAc-GlcUA of hyaluronic acid) being less than or equal to 0.1 and preferably less than or equal to 0.01.

Particularly preferred are the esters characterised by a degree of substitution comprised from 0.001 to 0.08, preferably from 0.002 to 0.03 or even more preferably from 0.003 to 0.01. The esters of the present invention have a hyaluronic acid molecular weight of between 10,000 and 100,000 D, even more preferably between 50,000 and 85,000 D.

The esters of the present invention have demonstrated an antiproliferative, antitumour and antimetastatic activity, surprisingly greater than that of butyric esters characterised by a higher degree of substitution. Said effect is observed both in *in vitro* experiments on tumour cell lines and in *in vivo* experiments on animal models of metastatic dissemination.

The esters of the invention are particularly active on primary and metastatic liver tumours and this activity seems to correlate with the levels of expression of CD44 on the tumor.

Therefore, one of the main aspects of the invention relates to the use of the aforedefined esters for preparing drugs targeted for the treatment of pathologies characterised by cell hyperproliferation as in the case of tumours, particularly indicated for primary or metastatic liver tumours. Of note, the esters of the invention, compared to those of the prior art US 6,140,313 are obtained under anhydrous reaction conditions. Under these conditions the esterification reaction proceeds in a more controlled manner than under not fully anhydrous conditions, hence esters with low degrees of substitution can be obtained by suitably varying

concentrations of the reagents. Indeed, in the presence of water, as in the case of the processes of the prior art, the acylating agent is partially hydrolysed and, because of the poor solubility of hyaluronic acid in organic solvents, acylation takes place in a heterogeneous phase: the ester groups are thus distributed in a non-uniform manner on the polymer matrix.

In the present invention, however, the process for preparing hyaluronic acid butyric esters takes place under anhydrous conditions in the homogenous phase, and is based on the use of hyaluronic acid in the form of a saturated quaternary nitrogen salt (such as an ammonium salt substituted with 4 alkyl or aryl residues) or an unsaturated quaternary nitrogen salt (such as an N-alkylpyridinium aromatic salt). A tetraalkylammonium salt of hyaluronic acid is preferably used, having good solubility in polar aprotic solvents. In particular, the tetrabutylammonium salt (HA-TBA) is preferred, obtained according to known methods, for example by passing the sodium salt of hyaluronic acid over an ion exchanger conditioned with TBA. A preferred ion exchanger is an amberlite resin IR 120 column in acid form, in which a 40% aqueous solution of TBA is recycled for at least 4 days at 40°C and subsequently washed with distilled H_2O until the pH is <10.

The hyaluronic acid quaternary salt is preferably dissolved in N,N-dimethylformamide (DMF) even if other solvents such as dimethyl sulfoxide (DMSO) or N-methylpyrrolidone can be used. In accordance with a preferred embodiment the process for preparing hyaluronic acid butyric esters with a degree of substitution less than or equal to 0.1 comprises the following passages:

a) dissolving a hyaluronic acid quaternary nitrogen salt at a concentration between 1 and 100 g/litre in a polar aprotic solvent, optionally heating to temperatures above 50°C,

b) preparing the acylating reagent by mixing, in a polar aprotic solvent, butyric anhydride and a 4-dialkylaminopyridine,

c) adding the acylating reagent to the hyaluronic acid salt solution under anhydrous conditions,

d) purifying the reaction product or alternatively converting the ester obtained into the corresponding sodium salt by means of acidification.

The acylating reaction is carried out under anhydrous conditions, for example

under nitrogen atmosphere. Preferred catalysts of the acylation reaction are 4-dialkylaminopyridines, among which 4-dimethylaminopyridine (DMAP) and 4-pyrrolidinopyridine (PPY).

The hyaluronc acid is preferably prepared as HA-TBA in anhydrous DMF, at concentrations between 1 and 100 g/litre, more preferably between 10 and 50 g/litre, preferably heated to 80°C until dissolved then left to cool to ambient temperature.

A quantity of butyric anhydride at a concentration comprised from 0.01 to 5 moles/litre (preferably from 0.1 to 2 moles/litre) and of dimethylaminopyridine (DMAP) in a molar ratio with the butyric anhydride between 0.1 and 10 (preferably between 0.5 and 2 or actually in an equimolar ratio with the butyric anhydride quantity), in a suitable volume of anhydrous DMF, are added drop-wise to the HA-TBA solution, under mechanical agitation, leaving it to react for at least an hour. The reaction is then stopped with distilled H_2O.

The tetrabutylammonium salt of the hyaluronic acid butyric esters (HA-But) is then converted to the corresponding sodium salt by acidifying the ester obtained with a diluted acid, preferably with 0.1 M HCl, then neutralised with a saturated solution of sodium hydrogen carbonate.

The aforedescribed conditions enable homogeneous synthesis, the esterification being therefore optimised in terms of yield, conversion efficiency and homogeneity of the product obtained. Moreover, under anhydrous conditions, the synthesis is more easily controllable, and depends mainly on the quantity of butyric anhydride added.

Therefore in these conditions, it is also possible to operate with limited amounts of butyric anhydride to obtain butyric acid esters with low degree of substitution, less than or equal to 0.01. In accordance with a preferred embodiment of the process, at point c) the acylating reagent is added in a quantity such that the molar ratio between butyric anhydride and the repeating disaccharide unit of hyaluronic acid is between 0.004 and 0.3, preferably between 0.008 and 0.1, or even more preferably between 0.01 and 0.03.

A further aspect of the invention relates to the possibility of obtaining pharmaceutical compositions containing, as active principle, a therapeutically

efficient quantity of a butyric ester of the invention in association with suitable pharmaceutically acceptable diluents and excipients. In this respect, in addition to the esters of the invention, said compositions can also contain other active principles and can be employed for oral use, in the form of granular powders, tablets, pills or gels.

The administration of the butyric esters of the invention can be systemic, oral, parenteral, topical or transdermal, they also being suitable for administration via the following routes: oral, intravenous, intraperitoneal, intraarticular, intramuscular, subcutaneous, rectal, intracavital (intravesicular or intravaginal).

The effective therapeutic dose varies preferably from 0.2 to 2 g/day for 1-15 days or, more preferably, from 0.3 and 1.5 g/day.

Toxicological tests have shown that the butyric esters of the invention are non-toxic and can therefore be used at the proposed doses or at even higher doses.

In the case of intravenous injections, the butyric ester is administered preferably at the dose of 0.2-50 mg/kg/day for 8-12 days. If injected intraperitoneally, the butyric ester is preferably administered as a solution, preferably in physiological solution, at a dose of 1-100 mg/kg/day (preferably 10-50 mg/kg/day) for 8-12 days. Finally, in the case of oral administration, the butyric ester is preferably administered at a dose of 300-500 mg/kg/day for 8-12 days.

As verified by using the technetium radiolabelled compound whose biodistribution was monitored scintigraphically, i.p. or s.c. administration is to be preferred for avoiding the compound segregating to the liver. The scintigraphic images collected 6 hours following s.c. administration indicate that a good 36% of ^{99m}Tc HA-But remains localised in the injection site suggesting that, according to the administration route applied, the drug can be delivered by the most suitable method to the various target organs: if i.v. administration is preferred for intrahepatic lesions, the s.c. route could prove to be more useful for the treatment of lesions in organs other than the liver, and the oral and rectal routes for the treatment of gastroduodenal and colorectal lesions respectively.

Moreover, it should be stressed that the new esters of the invention in which butyric acid is esterified with HA, are characterised by a high affinity for a specific membrane receptor (CD44). This specificity is therefore exploitable for

preferentially delivering the drug to target cells which express high levels of said receptor. Due to their specificity, the esters of the invention are an effective solution to the treatment of neoplastic lesions surrounded by normal tissue, such as intrahepatic lesions, with greatly reduced cell damage to normal cells as normally occurs with classical treatments with cytotoxic drugs.

The present invention therefore represents a new therapeutic approach for the treatment of intrahepatic lesions as the butyric esters of the invention, when administered intravenously, are accumulated preferentially in the liver and are internalised by the cells that overexpress the CD44 receptor. In contrast, administration of the compound by non-intravenous routes, such as subcutaneous, intraperitoneal, oral and rectal means, thus by-passing accumulation in the liver, allows a different distribution of the drug so that effective concentrations in the target organs are attained.

EXPERIMENTAL PART

Abbreviations used

$\%\ (w/w)_{butyric\ acid}$	% by weight of butyric acid $CH_3CH_3CH_2COOH$
$\%(w/w)_{butyrate}$ ester	% by weight of butyrate residue $CH_3CH_3CH_2COO$ in the
CE-UV	capillary electrophoresis with UV detector
Da	Dalton (unit of atomic mass) [equivalent to g/mole]
DMAP	4-(dimethylamino)pyridine
DMF	N,N-dimethylformamide
DS	degree of substitution
HA-Na	sodium salt of hyaluronic acid (sodium hyaluronate)
HA-But	butyrate esters of hyaluronic acid
HA-TBA	tetrabutylammonium salt of hyaluronic acid
Mdimer	average molecular weight of repeating unit (dimer)
M_w	weight average molecular weight
PTFE	poly(tetrafluoroethylene)
TBA	tetrabutylammonium
TBA-OH	tetrabutylammonium hydroxide

Materials

Hyaluronic acid, sodium salt, with a weight average molecular weight (M_w) of 85,000 Da (for HB10 synthesis) or 61,500 Da (for HB11, HE1, HE2, HE3 and HE4 synthesis) (*Bioiberica*) .

Butyric anhydride – tetrabutylammonium hydroxide, 40% aqueous solution by weight – anhydrous N,N-dimethylformamide (*Aldrich*). 4-(dimethylamino)pyridine (*Fluka*). Anhydrous sodium carbonate (*Riedel – de Haën*). N,N-dimethylformamide – methanol – diethylether - acetone – 37% hydrochloric acid by weight – silica gel (*Carlo Erba Reagents*).

The dialyses were undertaken using cellulose membrane tubes, with a molecular weight cut-off equal to 12,000-14,000 Da (*Spectrum* SPECTRA/POR 5), until the electrical conductivity of the solution decreased to below 3 µS/cm.

The total conversion of the products to sodium salts was achieved by passing the relative solutions through exchange resin *Amberlite IR-120 (plus) (Sigma-Aldrich)* in the form of sodium salt.

Example 1. Synthesis of the tetrabutylammonium salt of hyaluronc acid (HA-TBA).

The ion exchange resin *Amberlite IR-120 (plus)* in the form of tetrabutylammonium salt (TBA) was obtained from that in acid form (*Sigma-Aldrich)* by treatment with an aqueous 40 weight% solution of tetrabutylammonium hydroxide, recycled for 4 days through a jacketed column at 40°C.

HA-Na (500 g – 1.25 eq ≡ moles of repeating dimer units) was dissolved gradually in 10 litres of bidistilled water, maintaining the system under mechanical agitation at a temperature of ~ 25°C.

The solution was loaded and recycled in cocurrent (descending flow with velocity of ~ 100 ml/minute) for ~ 24 hours through a column containing *Amberlite IR-120 (plus)* ion exchange resin in the form of TBA salt (7 kg – 30.8 eq). The column was unloaded and the resin washed with bidistilled water (~ 13 litres) to recover all the residual product. Finally the solution was lyophilised to obtain 690 g (1.11 eq – yield 89%) of HA-TBA (M_{dimer} = 620.5 g/mol), a whitish solid to be stored under cool conditions.

Example 2. Synthesis of butyric esters of hyaluronic acid with degree of

substitution between 0.004 and 0.8.

Preparation of the hyaluronic acid butyric esters (HA-But) indicated by the abbreviations HB10, HB11, HE1, HE2, HE3 and HE4 are described herein. The synthesis was achieved at ambient temperature, under a head of nitrogen and under mechanical agitation.

5g ($8.1x10^{-3}$ eq ≡ moles of repeating dimer units) of tetrabutylammonium salt of hyaluronic acid (HA-But) were completely dissolved in 100 ml of anhydrous N,N-dimethylformamide (DMF) (*Fluka*) at 80°C, and the solution obtained was cooled to room temperature.

A 25 weight% solution of butyric anhydride in a quantity of 0.02 – 0.12 – 0.44 – 0.85 – 1.6 – 3.1 ml (respectively corresponding to 0.12 – 0.73 – 2.7 – 5.2 – 9.8 – 19 mmol for the synthesis of HB10 –HB11 –HE1 – HE2–HE3–HE4) (*Fluka*) and a 20 weight% solution of 4-(dimethylamino)pyridine (equimolar to butyric anhydride) (*Fluka*) in anhydrous DMF in a volume varying on the basis of the quantity of butyric anhydride, was added drop-wise over about 15 minutes to the HA-TBA solution. The reaction was left to proceed for a further 75 minutes under the same conditions, then stopped by the addition of distilled water.

The conversion from the tetrabutylammonium salt (TBA) to the sodium salt (Na) of the HA-But product was undertaken by acidification with 0.1 M hydrochloric acid (HCl) and neutralization with a saturated sodium hydrogen carbonate ($NaHCO_3$) solution.

The solution was then concentrated to about one fifth of the original volume with a rotary evaporator under reduced pressure; the polymer was then precipitated by pouring the concentrated solution into at least three volumes of acetone, then the precipitate was separated by filtration and dried under vacuum. The product obtained was then purified by dialysis of the aqueous solution of the crude solid against distilled water, using cellulose membrane dialysis tubes with a molecular weight cut-off equal to 12-14 kDa (*Visking*). The butyric esters of hyaluronic acid in the form of sodium salt were then obtained, by lyophilising, as white or whitish solids. The structure of the compounds was determined by proton magnetic resonance spectroscopy (^{1}H-NMR), undertaken on deuterated dimethylsulfoxide solutions (DMSO-d6) at 200 or 500 MHz using Bruker 200 AC or Varian Inova 500

spectrometers, respectively.

The ^{1}H-NMR spectrum of the HE2 compound (DS = 0.2) is given in figure 2: it was obtained by comparing the area of the signal relative to the methyl group (δ 0.83 ppm, triplet) of the butyrate residue with the area of the signal relative to the methyl group (δ 1.92 ppm, singlet) of the N-acetyl group belonging to the glucosamine residue of hyaluronic acid. Of note are the methylene group signals (δ 1.54 ppm, multiplet; δ 2.32 ppm, triplet) of the butyrate residue and those typical of hyaluronic acid (at δ values between 3.2 and 4.7 ppm).

Determination of degree of substitution of the esters obtained was performed by capillary electrophoresis with UV detection (CE-UV) (Figure 2), the degree of substitution in butyrate being as shown, by way of example, for the following products:

HB11: $DS_{butyrate}$ = 0.025 – %(w/w)$_{butyrate}$ = 0.53 – %(w/w)$_{butyric\ acid}$ = 0.54

(the average molecular weight of the dimmer repeating units is: M_{dimer} = 402.8 g/mole).

HB10: $DS_{butyrate}$ = 0.004 – %(w/w)$_{butyrate}$ = 0.08 – %(w/w)$_{butyric\ acid}$ = 0.08

The average molecular weight of the dimmer repeating units is therefore: M_{dimer} = 401.3 g/mole.

Analogously for other products obtained by the synthesis described in example 1, the following degrees of substitution were ascertained: 0.1 (HE1), 0.2 (HE2), 0.4 (HE3) and 0.8 (HE4).

Example 3. Scale-up of the synthesis of hyaluronic acid butyric esters.

Compound synthesis was achieved under conditions of mechanical agitation, at room temperature and under nitrogen flow. 55 g of HA-TBA, equal to $8.9x10^{-2}$ eq (moles of dimer repeating units), were completely dissolved in 3 litres of anhydrous DMF *(Fluka)*. The acylating agent was prepared by mixing 4 ml ($2.45x10^{-2}$ moles) of butyric anhydride (*Fluka*) and 3 g ($2.46 x10^{-2}$ moles) of DMAP (*Fluka*) in 200 ml of anhydrous DMF.

The butyric anhydride solution was added drop-wise to the HA-TBA solution in DMF over a period of about 25 minutes. The reaction was allowed to proceed for a further 65 minutes.

The tetrabutylammonium sodium salt of HA-But was then converted to the

corresponding sodium salt by adding distilled water Milli-Q (~6 litres), acidifying with 0.1 M HCl and neutralizing with 1 M NaOH. The polymer was then purified by dialysis against Milli-Q distilled water (14 x 30 litres/each) in a tube with cellulose membrane (molecular weight cut off: 12-14 kDa; *Visking*) and then through a column containing 500 ml (0.95 eq) of Amberlite IR-120 (plus) ion exchange resin in the form of sodium salt (*Aldrich*) at room temperature for 1 hour. The final quantity of the HA-But compound, obtained as the sodium salt by cold lyophilization (called HB4), was 34 g (8.4x10-2 eq; 94% yield).

The butyric acid content of the ester thus prepared was determined by capillary electrophoresis with UV detector using an Applied Biosystems HPCE Model 270A-HT, with silica capillary column (72 cm x 50 µm I.D. x 375 µm O.D.; *Supelco*). The analytical conditions used were: temperature 27°C, pressure 16.9 kPa (1.5 s), voltage 20 kV, UV detector at 195 nm, buffer Borax 50 mM (pH=9.5) (*Sigma*), wash solution 0.1 M NaOH. The calibration curves were obtained by using the following graduated progression: 0.001, 0.005, 0.05, 0.1% (v/v) of butyric acid in Milli-Q (Millipore) distilled water. The butyric ester was then hydrolysed in 0.1 M NaOH at ambient temperature for 2 hours and the reaction was stopped by neutralizing with 0.1 M HCl. The final concentration of HB4 was 2 mg/ml.

Measurement of the butyric acid was undertaken in triplicate and the limits of detection (L.O.D) and quantification (L.O.Q) were calculated as follows:

L.O.D. = 3.33*S/m; L.O.Q. = 10*S/m; where S is the standard deviation of the interception of the 3 calibration curves and m is the average of the gradient of the 3 calibration curves.

In the electropherogram of the hydrolysed polymer (figure 2) the butyric acid peak showed a migration time of 20.55 min, while the hyaluronic acid peak showed a migration time of 15 minutes. The L.O.D value was calculated as 4.43 µg/ml and that of L.O.Q was 13.30 µg/ml. By analysing with CE-UV, HB4 was therefore found to have a DS of 0.062, a butyrate % (w/w) of 1.33 and a molecular weight, with reference to the dimer unit, of 405.36 g/mole.

Example 4. In vitro evaluation of the biological activity of the esters of the invention.

The antiproliferative effect of graduated doses of the esters prepared as in

example 2 were evaluated on a human colon carcinoma line (HT29) and on a human hepatocarcinoma line (HepB3) (Figure 3, panel A and B, respectively) acquired from the *American Tissue Culture Collection.*

The two cell lines were seeded at a density of 1000 cells per well in a 96-well tray in RPMI 1640 medium to which was added 10% (v/v) fetal bovine serum. After 24 hours of adhesion time, the seeding medium was removed and replaced with that containing the esters of the invention. The cells were maintained in the presence of graduated concentrations of hyaluronic acid (HA) (range: 0.016 – 4 mg/ml), butyrate esters (HE3/T, HB4, HB10 and HB11) (range: 0.016 – 4 mg/ml) or sodium butyrate (range: 0.016 – 4 mM) for 6 days. The experiments were conducted at least twice, each experimental condition being set up in 8 replicates. The antiproliferative effect was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide (MTT) method, the formazane precipitates thus obtained being resuspended in dimethyl sulfoxide and measured spectrophotometrically at a wavelength of 540 nm. Wells containing all the reagents except cells were used as blanks. The effect of the compounds under examination was expressed as percentage inhibition compared to the control (consisting of cells maintained in culture medium alone).

All the butyric monoesters tested showed a dose-dependent inhibitory effect which was clearly greater than that exerted by the active principle alone. In particular, the compound with the lowest degree of substitution (0.004 – HB10) was found to induce, in the HT29 cells, a 50% inhibition at a concentration (in terms of butyrate) about 60 times lower than that required to obtain the same effect with the active principle (figure 3A).

All the butyric monoesters tested also showed a dose-dependent inhibitory effect on Hep3B cells which was clearly greater than that exerted by the active principle alone (figure 3B). In these cells in particular, the compound with the lowest degree of substitution was found to be capable of inhibiting cell growth by 50% at a concentration (in terms of butyrate) about 120 times lower than that necessary to obtain the same effect with the active principle.

Example 5. *In vitro* evaluation of the antiproliferative effect of the esters of the invention as a function of the CD44 hyaluronic acid receptor expression.

Expression of the CD44 receptor in two hepatocarcinoma cell lines Hep3B and HepG2 was evaluated by flow cytometry using a murine monoclonal antibody against human CD44 (clone 5F12; *Neo Markers, Labvision, Fremont*, CA) and a goat anti-mouse secondary antibody conjugated with FITC (*Sigma, St. Louis, MO*) in accordance with known techniques. The negative control was obtained by incubating the specimen with the secondary antibody alone. Following immunofluorescent labelling, the cells were incubated with propidium iodide (5 g/ml), RNase (10 kU/ml), (*Sigma*), and Nonidet P40 (0.005%) and fluorescence was measured with a FAC Scan flow cytometer (*Becton Dickinson, San Jose, CA*) equipped with argon laser. The fluorescent signal was acquired in both linear mode and logarithmic mode. The cytometric examination demonstrated that only 18% of the HepG2 cells express CD44 receptors on their cell surfaces, in contrast to 78% of HepB3 cells (figure 5A).

As shown in figure 4, despite the differing expression of CD44, the butyric ester HB10 was able to inhibit cell proliferation in a dose-dependent manner in both cell lines. In HepB3 cells, after 6 days' treatment, HA-But at the highest concentration (4 mg/ml corresponding to a 1 mM concentration of butyric acid) induced almost complete inhibition of cell growth (90%), while in the HepG2 cells, which express CD44 at a low percentage, only a moderate inhibition was observed (60%). This result suggests however that even in the presence of a low percentage of cells expressing the HA receptor, HA-But is able to considerably reduce cell growth. Indeed, in both cell lines HA-But was found to be much more effective than sodium butyrate with an approximately 10 fold reduction in IC_{50} value compared to that observable with the active principle alone (2.1 vs 0.12 mM in HepG2 cells and 3 vs 0.31 mM in HepB3 cells) suggesting that the use of a suitable carrier can significantly increase biological activity of the sodium butyrate without negatively influencing its biological activity due to the presence of a bulky molecule such as that of HA.

Example 6. Evaluation of binding kinetics with labelled butyric esters. *In vitro* and *in vivo* studies.

Binding kinetics of the esters of the invention to the HepG2 and HepB3 hepatic cell lines was evaluated by cell uptake of technetium labelled butyric ester (^{99m}Tc-

HA-But). In this respect, HA and HA-But were labelled with technetium-99m (^{99m}Tc), the most frequently used radioisotope for radiodiagnostic purposes, to investigate by known methods the *in vivo* biodistribution of a given compound. The stability of the labelled compounds was determined in the presence of rat serum for at least 6 hours, during which period ^{99m}Tc-pertechnetate formation was not observed, confirming the stability of the metallo-polymer complexes.

HepG2 and HepG2 cell lines were used for the *in vitro* experiments, incubated with purified labelled HA-But and diluted with native unlabelled HA to a final concentration of 5 mg/ml, at 37°C. After incubating with ^{99m}Tc-HA-But, the total bound radioactivity was measured by means of a γ-counter. In parallel, to verify the specific bond between HA and the CD44 membrane receptors, the cells were pre-treated with an unlabelled HA solution (50 mg/ml) for 4 hours at 37°C.

As demonstrated in figure 5B, cell uptake experiments conducted using ^{99m}Tc-HA-But confirmed what had already been observed by flow cytometry. Moreover, the time-dependent experiments (figure 5C) indicated that the two cell lines had differing binding kinetics: the binding activity of HepB3 increased in a time-dependent manner attaining plateau about 6 hours after the start of treatment, whereas the HepG2 cells demonstrated a limited binding activity which had already begun to decrease after 4 hours.

Regarding the *in vivo* experiments, the scintigraphic images showed that following i.v. administration, the labelled compound accumulated preferentially in the liver in a manner analogous to that observed with native hyaluronic acid. This result is not surprising considering that the circulating HA is physiologically degraded by the hepatic sinusoidal endothelial cells via CD44. Moreover, *ex vivo* analysis demonstrated considerable accumulation of ^{99m}T-HA-But in the spleen, an unsurprising result considering the role of the spleen in HA degradation. By considering the absolute quantity of ^{99m}T-HA-But per gram of tissue (so as to consider the actual dimensions of the organ under discussion) instead of the percentage of accumulated radioactivity in a given organ, it has been observed that the spleen accumulated 40.8 µCi per g of tissue compared with the liver, which accumulated 55.3 µCi per gram of tissue.

As expected the high accumulation of labelled compound observed in the liver

after i.v. administration was reduced considerably when ^{99m}T-HA-But was administered i.p. or s.c. indicating that i.p. could be a particularly useful administration method for avoiding HA segregating to the liver. The scintigraphic images, gathered 6 hours following s.c administration also showed that a good 36% of ^{99m}Tc HA-But remains localised in the injection site suggesting that the different administration routes could deliver the drug in the most appropriate manner: the i.v. administration route could be the one of choice for the treatment of intrahepatic lesions, while the s.c. method could be much more useful for treating more easily accessible lesions for which a slow release could be more appropriate.

Example 7. *In vivo* evaluation of acute and subacute toxicity of the esters.

The acute toxicity of the butyric ester HB10 prepared as described in example 2, and administered intravenously was firstly evaluated. The lethal dose for 50% of the animals treated (LD_{50}) was found to be greater than 4.8 µmol/mouse.

Table 1. Acute toxicity of *in vivo* treatment with HB10 administered intravenously.

HA-But dose (µmoles/mouse)	Administration route	Mortality: number dead/ total number	Body weight variation (g) ♠
2.4 (µmoles)	i.v.	0/5	+0.70
4.8 (µmoles)	i.v.	0/5	+0.40
12 (µmoles)	i.p.	0/5	+0.70
24 (µmoles)	s.c.	0/5	+0.60

In the experiment presented in table 1, groups of 5 female Swiss mice weighing 22-22 g were treated intravenously with HB10 at the concentrations indicated. The average difference in body weight (♠) is expressed as the difference in weight from the measurement taken 24 hours before and 6 days after treatment. At the end of the treatment the animals were observed for a further 30-day period. No deaths were observed with any of the doses employed.

Table 2. Subacute toxicity of *in vivo* treatment with HB10 administered intraperitoneally or subcutaneously.

HA-But dose (μmoles/mouse/day)	Administration route (days)	Mortality number dead/ total number	Difference in body weight (g)♠
12 (μmoles)	i.p. (10)	0/10	+0.40
4.8 (μmoles)	s.c. (25)	0/15	+0.30

In the experiment presented in table 2, groups of 10-15 female BD2F1 mice weighing 10-22 g were treated with HB10, by the administration route indicated, at concentrations of 12 μmoles /mouse i.p. or 4.8 μmoles /mouse s.c. The average difference in body weight (♠) is expressed as the difference in weight from the measurement taken 24 hours before treatment and at the end of treatment. At the end to the treatment the animals were observed for a further period of 30 days. No deaths were observed with any of the doses used.

The absence of toxicity on the part of butyric ester was confirmed by the long-term survival of the animals (up to 90 days) and by the minimum weight differences (table 2).

Example 8. *In vivo* activity of the butyric esters of the invention: evaluation of antimetastatic activity and effect on survival.

Since the *in vivo* biodistribution studies conducted with technetium labelled HA-But have demonstrated that, after i.v. administration, the preferential distribution of the compound is in the liver, it was decided to make use of this peculiarity for therapeutic purposes by investigating the antitumour activity on animal models of hepatic neoplasias. In order to have an *in vivo* model similar to that observed clinically, an animal model was therefore chosen which provides for an intrasplenic inoculum of Lewis LL3 carcinoma or B16-F10 melanoma cells, both able to spontaneously colonise in the liver. The experimental model developed and used in this series of experiments is therefore different from that which provides for colonisation of the liver by tumour cells directly injected into the circulation and uses a mechanism of metastatic invasion more similar to that observed clinically.

Animals. In the experiments described, mice strains C57BL/6, C57BL/6xDBA/2F1 (BD2F1) and CBA/Lac were used acquired from *Harlan & Nossan, San Giovanni*

al Natisone, Udine, Italy, aged from 3 to 6 months and weighing 18-20 g. All the experiments were conducted in accordance with the regulations currently in force in Italy (DDL 116, 21/12/92) and The Guide for the Care and Use of Laboratory Animals. (DHHS Publ. No [NIH], 86-23, Bethesda, MD: NIH, 1985).

Cells. The Lewis lung carcinoma (LL3) line was obtained from the *Tumor Depository Bank, NCI, NIH (Bethesda, USA)* and maintained in culture by means of serial passages in accordance with NCI protocols: $1x10^6$ viable tumour cells obtained from a single suspension and prepared by dissecting a primary tumour grown in an animal donor for 2 weeks, were injected i.m. into the left paw of female mice, strain C57BL/6. The tissue fragments obtained were filtered through a double layer of sterile gauze, centrifuged at 200 x g for 10 minutes and the cells thus obtained were resuspended in an adequate volume of sterile buffer solution. The cells were then counted using the Trypan Blue exclusion method.

B16-F10 malignant melanoma cells, originally obtained from the *Tumor Depository Bank, NCI, NIH (Bethesda, USA),* were cultivated in MEM medium to which were added 10% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamine (100x), 1% standard antibiotic solution, 2% $NaHCO_3$ and 1% glucose, and were maintained in an incubator in a humid atmosphere with 5% CO_2 at 37°C. The tumour cells thus obtained were then inoculated intrasplenically in a sterile environment, into female strain BD2F1 mice previously anaesthetised by i.p injection with Zoletil (70 mg/kg) (*Virbac srl, Milan, Italy*). About $2x10^5$ Lewis LL3 carcinoma cells or $1x10^5$ B16/F10 melanoma cells were inoculated after suitably dissolving in a Matrigel solution (150 µg/ml, *Beckton Dickinson*) to enable a better implantation in the organ. 4 days after the inoculation, (required to allow the tumour cells to implant), groups of 8 animals were each treated i.p. or s.c. with 0.6 µmoles (250 µl/mouse) of HB10, prepared as described in Example 2, in absolutely non-toxic doses as demonstrated in previous acute and chronic toxicity studies, and then treated daily until day 10. The control animals were instead treated i.p or s.c. with an equal volume of physiological solution. 15 days after the inoculation of LL3 cells and 21 days after inoculation of B16/F10 cells the animals were sacrificed, their livers explanted and the formation of metastases evaluated by using a stereo-microscope with

calibrated grid.

Statistical analysis. The experimental data were analysed with the aid of suitable software and the statistical significance of the effect of the treatment compared with the control was evaluated by applying the Student test, whereas the Fisher exact test was used to evaluate the effect of HA-But on metastases formation. Survival of the animals was instead evaluated by Kaplan-Meier analysis. Only probability values of less than 0.05 were considered statistically significant.

Possible toxic effects of HA-But on hepatic parenchyma were evaluated microscopically in 4µm sections stained with Cajal-Gallego, obtained from samples of liver fixed in 10% formalin and enclosed in paraffin.

The effect of HB10 on the survival of tumour bearing animals was investigated in a parallel series of experiments in which the female BD2F1 mice, inoculated intrasplenically with $1x10^5$ B16/F10 melanoma cells, were treated s.c. with 0.15 µmoles (125 µl/mouse/day) of HA-But from day 4 after inoculation to day 32 (the day on which the death of the first control animal was observed), with a further i.p administration of 12 µmoles/mouse on days 4, 11, 18, 25 and 31. Survival of the animals was monitored until the death of the last control animal.

The effect of HA-But administered i.p or s.c at a concentration of 0.6 µmol on the formation of hepatic metastases is given in table 3.

In the model with the LL3 cell inoculum, 15 days after implantation, HA-But treatment was able to significantly reduce liver colonisation so that 87% (13/15) of the treated animals were free of microscopically verifiable metastasis and only one animal per administration method group (i.p. or s.c.) presented metastatic foci. In contrast, in the control group only 13% (2/15) of the animals were free from metastases. A similar response was also observed in the mice inoculated with the B16-F10 melanoma cells. 21 days after implantation, all the animals treated with the butyric esters of the invention (HA-But or HB10) were free of microscopically visible metastases, while all the control animals presented numerous metastatic lesions.

Table 3. Effect of i.p. or s.c. HB10 treatment for 7 days on the formation of hepatic metastases induced by intrasplenic implantation of Lewis LL3 carcinoma or B16-F10 melanoma cells

		LL3		B16/F10	
Administration route	Treatment	Number of liver metastases	Number animals without metastases	Number of liver metastases	Number of animals without metastases
s.c.	control	>10	1/7	>10	0/7
	HA-But	< 5	6/7*	-	6/6*
i.p.	control	>10	1/8	>10	0/8
	HA-But	< 5	7/8*	-	7/7*

*P<0.05, compared to control (Fisher exact test).

As demonstrated in figure 6, which presents the histological examination of the hepatic parenchyma, HB10 treatment did not alter its morphology. Worthy of note is the observation that, although the administration methods used were those least indicated by the biodistribution analysis, but were used because of their greater practicality in the murine model, HA-But was found to be very effective for inhibiting hepatic metastases formation which suggests that treatment for a sufficiently long period could enable HA-But to be effective independently of its means of administration.

Kaplan-Meier analysis (figure 7), applied to evaluate the effect of the treatment with HA-But on survival of the animals inoculated intrasplenically with B16/F10 melanoma cells (model chosen for its particular aggressiveness and for its high percentage of CD44 receptor positivity), has highlighted that the animals treated with even low doses of HA-But (4.8 μmoles/mouse/day) had a statistically significant survival period compared to control animals: after 90 days from the start of the experiment 80% of the animals treated with HA-But were still alive compared to the 27% observed in the control group. It should be emphasised that all the animals treated with HA-But were also found to be still alive 5 months after the start of the experiment, this being a substantially long period considering the average lifespan of these animals and the particular aggressiveness of the cell model used.

The results obtained until now seem to indicate that the esters of the invention containing butyric acid (the smallest of the histone-deacetylase inhibitors) and HA, can represent an excellent alternative for the stabilisation and delivery of butyric

acid, and a promising approach for the treatment of intrahepatic lesions. Without doubt the use of histone-deacetylase inhibitors as antitumour agents is not new and some of them (phenylbutyrate, suberoylanilide hydroxamic acid, MS-275, depsipeptide) are currently being studied in phase I and II clinical trials for the treatment of solid tumours and lymphomas. It is important to note however that none of these drugs fully satisfies one of the principle requirements of oncology therapy: the selective administration of antitumour molecules directly to the target cells.

With this in view, HA-But, whose carrier molecule (HA) is characterised by a high affinity for a specific membrane receptor known to be overexpressed by the tumour cells, could constitute a turning point in the treatment of primary and metastatic intrahepatic lesions of other origins.

Example 9. Activity of butyric acid esters on the metastasisation of tumour cells derived from the TLX5 lymphoma.

In parallel with the previous studies, the pharmacological activity of hyaluronic acid butyric esters prepared as in example 2 was also evaluated on another experimental tumour model: a murine lymphoma (TLX5) that grows in ascites form in the peritoneum. The single subcutaneous treatment with HB10 at a dose of 12 µmoles/mouse effected 24 hours after the tumour inoculation, significantly reduced the number of tumour cells present in the peritoneum at the moment when the animal was killed. As shown in table 4, said effect was not however accompanied by a significant increase in survival time of the treated animals.

Table 4. Effect of *in vivo* treatment with HA-But on survival time, on the number of tumour cells and on their dissemination in the brains of TLX5 lymphoma bearing mice.

Treatment (µmoles/mouse)	Survival ♣ (days)	Peritoneal ascites ♦ (number of cells x10^6)	Brain bioassay ♠ survival (days)
Controls	10.4±0.6	916.0±129.4	14.2±1.1
Habut (12)	9.0±1.3	507.5± 39.5*	15.5±0.5

*P<0.05, compared to control (T-test).

For the experiments presented in table 4, groups of 10 male mice of strain CBA/Lac were inoculated i.p. with 100,000 TLX5 lymphoma cells and treated 24 hours after tumour implantation with HB10 at the single dose of 12 μmoles/mouse (0.5 ml s.c.). In 5 animals per group the survival time (♣) was evaluated , while the remainder were sacrificed on the 8th day for the peritoneal tumour cell count (♦). The brains of these animals were also removed (preferential site of metastasisation for this experimental model) in order to re-inoculate it subcutaneously into healthy animals from the same strain in which the survival time (♠) was determined. The data were expressed as median±s.e.

When the HB10 was administered directly in the peritoneum in order to ensure direct contact between tumour cells and the compound, then repeated for 7 consecutive days at doses of 6 and 12μmoles/mouse/day, a statistically significant dose-dependent reduction of tumour load was observed, not being accompanied however by a concurrent increment in the survival time of treated animals (table 5).

Table 5. Effect of repeated *in vivo* treatments with HA-But on survival time, and on the number of tumour cells in TLX5 lymphoma bearing mice.

Treatment (μmoles/mouse)	Survival ♣ (days)	% reduction tumour load	Peritoneal ascites ♦ (number of cells x10^6)
Controls	9.6±0.2		689.0±126.0
Habut (6)	10.4±0.8	32	471.3± 48.1*
Habut (12)	11.6±0.6	69	216.3± 48.5*

*P<0.05, compared to control (T-test).

In the experiments described in table 5, groups of 8 male mice of strain CBA/Lac, inoculated i.p. with 100,000 TLX5 lymphoma cells, were treated 24 hours after tumour implantation with HB10 at doses of 6 or 12 μmoles/mouse (250 μl or 500 μl i.p.) for 7 consecutive days. Survival time (♣) was evaluated in 5 animals per group, while in the remaining animals, on the 8th day the count of peritoneal tumour cells was undertaken (♦). The data are expressed as median±s.e.

At the higher dose (12 μmoles/mouse) the butyl esters of the invention induced a 69% reduction in tumour load corresponding on average an additional 2 days of life for the treated animals compared to controls. Even this reduction, however,

does not prevent metastatic dissemination of leukaemic cells to the meninges, as indicated by the bioassay data which did not show a difference in survival between the treated and control mice. In this regard it should be noted that for all the experimental tumour models in ascites form, a direct correlation exists between the number of cells present in the peritoneum of the animal and its death, determined by the replication time which is specific for each type of neoplasia.